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DNA fusion gene vaccination mobilizes effective anti-leukemic cytotoxic T lymphocytes from a tolerized repertoire

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Abstract

The majority of known human tumor-associated antigens derive from non-mutated self proteins. T cell tolerance, essential to prevent autoimmunity, must therefore be cautiously circumvented to generate cytotoxic T cell responses against these targets. Our strategy uses DNA fusion vaccines to activate high levels of peptide-specific CTL. Key foreign sequences from tetanus toxin activate tolerance-breaking CD4⁺ T cell help. Candidate MHC class I binding tumor peptide sequences are fused to the C terminus for optimal processing and presentation. To model performance against a leukemia-associated antigen in a tolerized setting, we constructed a fusion vaccine encoding an immunodominant CTL epitope derived from Friend murine leukemia virus gag protein (FMuLV_{gag}) and vaccinated tolerant FMuLV_{gag}-transgenic (gag-Tg) mice. Vaccination with the construct induced epitope-specific IFN- γ -producing CD8⁺ T cells in normal and gag-Tg mice. The frequency and avidity of activated cells were reduced in gag-Tg mice, and no autoimmune injury resulted. However, these CD8⁺ T cells did exhibit gag-specific cytotoxicity *in vitro* and *in vivo*. Also, epitope-specific CTL killed FBL-3 leukemia cells expressing endogenous FMuLV_{gag} antigen and protected against leukemia challenge *in vivo*. These results demonstrate a simple strategy to engage anti-microbial T cell help to activate epitope-specific polyclonal CD8⁺ T cell responses from a residual tolerized repertoire.

Keywords

T cells; Tolerance; Transgenic mice models; Tumor immunology; Vaccination

Introduction

There is clear clinical evidence from recipients of allogeneic hematopoietic transplants that CD8⁺ T cells reactive with leukemic cells can be curative [1]. To build on this success, passive transfer of autologous, allogeneic or engineered CD8⁺ T cells against a range of target antigens

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is now being tested [2]. Such studies should provide proof of principle and validation of selected targets, but the technical demands of this approach are high, and questions still remain regarding the nature of the cell to be transferred and the ability of cells extensively expanded *in vitro* to survive, divide, and migrate *in vivo* [2]. An alternative approach is to actively induce an immune response in the tumor-bearing host, but the ability to achieve this has been questioned [3]. A major obstacle derives from the limited capacity of patients to respond if the available repertoire has been weakened by the processes of tolerance. However, the fact that cytotoxic CD8⁺ T cells (CTL) can be induced *in vitro* against a range of normally expressed peptides, and that these can kill tumor cells, suggests that deletional tolerance is incomplete in humans [4]. In addition, there is clinical evidence that tumor-specific T cells can be induced *in vivo* against self proteins, although whether the magnitude and avidity of the responses will be sufficient to control tumor growth is less clear [5].

For candidate tumor antigens that are self proteins, it remains likely that the highest-avidity cells have been deleted and the remaining potentially reactive repertoire is subject to peripheral tolerance mechanisms [6-8]. Therefore, the challenge is to design vaccines that can overcome the tolerogenic pressure on the residual repertoire. Once rescued from a tolerant state, it is essential that these T cells, likely of intermediate or low avidity, can recognize and kill tumor cells expressing the targeted antigen. Numerous vaccination strategies are being actively pursued [9]. We have focused on genebased vaccines since they provide a flexible platform for expressing antigens together with molecules able to direct and amplify immunity [10]. Clinical use of optimized DNA vaccines could follow from murine studies but translation to human subjects has had to address two problems. The first was a concern that stimulation of innate immunity via backbone CpG sequences might fail due to differences in expression of TLR9 between mice and humans [11]. However, DNA vaccines retain immunogenicity in TLR9-knockout mice [12], pointing to TLR9-independent pathways of immune activation. The second obstacle reflected the dependence of transfection level on the volume injected, which posed problems for scaling up from mice to human subjects [13-15]. Adequate antigen levels are required especially at boosting, and difficulty in achieving high transfection efficiencies could fail to boost responses in larger hosts [15]. To overcome these problems, and to add an inflammatory stimulus, viral or bacterial vectors are being examined [16]. New physical methods (e.g. electroporation) also appear to be effective and have the advantage, compared to pathogen-based vectors, of not inducing blocking immunity against the vector [13, 14, 17]. Electroporation is highly effective in large animals [18] and we are currently testing it in the clinic, with no ill effects noted. Importantly, DNA vaccines appear safe, and, based on their potential and safety profile, are now in pilot clinical trials [19, 20]. We have developed DNA fusion vaccines which encode two functional elements: one to stimulate CD4⁺ T cell help using a microbial sequence, in our case a domain (DOM) of fragment C (FrC) from tetanus toxin (TT), and the other consisting of a candidate tumor-specific MHC class I-binding peptide, fused to the C terminus (p.DOM-peptide) [21]. The microbial component (DOM) was minimized to reduce the potential for inclusion of immunodominant CTL epitopes that could compete with the tumor-specific peptide [22]. Crucially, this design engages CD4⁺ T cells from the non-tolerized anti-microbial repertoire. Induced responses against the fused MHC class I-binding epitope were shown to depend on T cell help [23], a key requirement for the induction and maintenance of effective CD8⁺ T cell immunity [24-26]. The ability of the cellular machinery to dissect out candidate tumor epitopes from the backbone sequence of the encoded antigen presents a limiting factor for presentation and immune induction [27]. We have overcome this by cutting out the tumor-derived CTL epitope, placing it downstream of the microbial DOM sequence to ensure optimal processing and presentation [27]. We have demonstrated that this strategy activates high levels of epitope-specific CD8⁺ T cells and establishes a durable memory response, leading to protective immunity in pre-clinical tumor models [22, 27-29]. These studies also revealed that it was possible to mobilize CD8⁺ T cells

reactive with a male (H-Y)- specific epitope from a profoundly tolerized repertoire in male mice [28]. However, the induced CD8⁺ T cells were unable to kill male splenocytes [28].

To model the performance of this strategy against a leukemia-associated antigen which is also a self protein, we have focused on the immunodominant CTL epitope (gag₈₅₋₉₃) derived from the Friend murine leukemia virus (FMuLV) gag protein (FMuLV_{gag}) expressed by FBL-3, an FMuLV-transformed mouse leukemia [30,³¹]. Previously we generated a transgenic mouse model in which FMuLV_{gag} is expressed in the liver as a normal self protein (gag-Tg mice). Tolerance to gag was evident from the fact that, in contrast to normal C57BL/6 (B6) mice, “conventional” vaccination with FBL-3 tumor cells failed to generate a CD8⁺ T cell response to FMuLV_{gag} or to induce resistance to tumor [32]. In the current study, we used the tolerant transgenic mice to assess the ability of a novel DNA fusion gene vaccine to induce effective immunity against a tumor antigen that is also expressed by normal cells. For disseminated leukemia, the goal can be limited to induction of CTL, since, once induced, CD8⁺ T cells should be able to find and destroy accessible targets. Here we show that a DNA fusion vaccine containing an epitope from the targeted tumor antigen and heterologous helper epitopes can break tolerance and activate low-avidity gag₈₅₋₉₃-specific CTL in mice expressing gag protein as a self antigen, with no evidence of autoimmune injury. The induced CTL acquired cytotoxic function in vivo and provided some protection against leukemia in vaccinated transgenic mice. This model of continuous expression of a leukemia-associated antigen in normal tissues has relevance for the clinic and reveals that DNA vaccination can activate surviving epitope-specific CTL for focused immune attack against leukemic cells.

Results

Expression of gag RNA in liver and thymus of gag-Tg mice and leukemic cells

In the gag-Tg model the antigen is expressed not only in the liver but also in the thymus, leading to tolerance in the CD8⁺ T cell repertoire [32,³³]. However, the level of gag antigen expression in these transgenic tissues compared to FBL-3 tumor cells is unknown. As a measure of expression, we used quantitative RT-PCR (qRT-PCR). Surprisingly, both thymus and liver biopsies from gag-Tg mice expressed higher levels of gag RNA compared to FBL-3 tumor cells (about twofold and threefold, respectively) (Fig. 1). Although this is at the RNA level only, it is indicative of higher levels of antigen expression. No amplification was detected in control reactions lacking cDNA (data not shown). No gag transcript amplification was detected using cDNA from EL4 cells or liver/thymus biopsies from WT mice (data not shown).

DNA vaccination induces gag₈₅₋₉₃-specific CD8⁺ T cells in WT and gag-Tg mice

In order to investigate whether our vaccination strategy could overcome immune tolerance in this model, we constructed a DNA fusion vaccine encoding the first domain (DOM) of FrC from TT linked to the immunodominant H-2D^b-restricted gag₈₅₋₉₃ peptide (p.DOM-gag). DNA vaccine design is indicated in Fig. 2. The ability of the p.DOM-gag DNA vaccine to induce CD8⁺ T cell responses to gag₈₅₋₉₃ was assessed by vaccinating WT and gag-Tg mice. For comparison, control groups were immunized with irradiated FBL-3 cells, known to induce a readily detectable protective CD8⁺ T cell response specific for the gag₈₅₋₉₃ epitope in WT mice [30,³¹]. T cell responses in the spleen were measured immediately *ex vivo* by ELISPOT assay on day 36. Vaccination of B6 mice with p.DOM-gag induced gag₈₅₋₉₃- specific T cell responses as measured by ELISPOT assay for the production of IFN-γ (Fig. 3A), with frequencies similar to those observed in FBL-3-vaccinated mice. The control DNA vaccine (p.DOM) generated no gag₈₅₋₉₃-specific responses (Fig. 3A). CD4⁺ Th cell responses against the ‘promiscuous’ MHC class II binding peptide p30, embedded in the FrC domain (DOM), were also detected in mice vaccinated with either DNA vaccine (p.DOMgag or p.DOM), but not in mice immunized with irradiated FBL-3 cells, in which the low frequency of IFN-γ-

producing cells detected was equivalent to background levels without peptide stimulation (Fig. 3A).

Gag-Tg mice were also tested for the ability to respond to the p.DOM-gag DNA vaccine. This vaccine induced robust gag₈₅₋₉₃-specific T cell responses, as monitored by the *ex vivo* IFN- γ ELISPOT assay (Fig. 3B), although the frequency of responding cells was ~2.5-fold lower than in WT mice (Fig. 3C). By contrast, immunization with irradiated FBL-3 tumor cells failed to induce detectable gag₈₅₋₉₃-specific T cell responses in gag-Tg mice (Fig. 3B), as previously reported [32]. A non-specific background response of cells producing IFN- γ was observed in mice after immunization with FBL-3, but this response was detected independent of stimulation with any peptide. It may be the consequence of an inflammatory response resulting from the injection of 1×10^7 irradiated tumor cells 8 days prior to obtaining the spleen, as a non-specific response of similar strength was also observed in WT mice immunized with the cellular vaccine (Fig. 3A).

The control p.DOM vaccine again induced no gag₈₅₋₉₃-specific responses, although CD4⁺ Th cell responses to the p30 peptide were detected in these animals, as well as those immunized with p.DOM-gag (Fig. 3B). Since the CD4⁺ T cell response to p30 is derived from the unmanipulated mouse repertoire, this was used as an overall indicator of the DNA vaccine performance, which appeared effective in both WT and gag-Tg mice (Table 1). A survey of larger numbers of individual mice was then carried out, which demonstrated that although p.DOM-gag activated a lower frequency of gag₈₅₋₉₃-specific T cells in gag-Tg mice compared to WT mice (Fig. 3C), the number of animals responding was comparable between strains: 92% (12 of 13) of gag-Tg mice and 100% (11 of 11) of WT mice (Table 1). The efficacy of the vaccine was further assessed by flow cytometric analysis to directly quantify the number of gag₈₅₋₉₃-specific CD8⁺ T cells present in splenic lymphocytes, using anti-IFN- γ (intracellular) and anti-CD8 (surface) antibodies (Fig. 3D). Responses were again clearly evident in WT and gag-Tg mice, and confirmed the lower frequency of responding cells in the latter (Fig. 3D).

Avidity of induced gag₈₅₋₉₃-specific CD8⁺ T cells in WT and gag-Tg mice

The frequency of gag₈₅₋₉₃-specific cells elicited in gag-Tg mice is low compared to WT mice, probably due to central and peripheral tolerance mechanisms which could potentially have deleted a fraction of the repertoire, particularly the high-avidity gag₈₅₋₉₃-specific CD8⁺ T cells. To address the question of avidity, we compared the frequency of CD8⁺ T cells from WT and gag-Tg mice that responded to varying concentrations of gag₈₅₋₉₃ peptide in an IFN- γ ELISPOT assay. WT and gag-Tg mice were vaccinated with either p.DOM-gag or the control DNA vaccine and ELISPOT responses were measured at day 36, as described above. Gag₈₅₋₉₃-specific CD8⁺ T cells elicited in the gag-Tg mice had about tenfold lower avidity compared to those from WT mice when tested against a range of gag₈₅₋₉₃ peptide concentrations *ex vivo*, with responses first detectable at 1 log higher peptide concentration (0.01 IM vs. 0.001 IM) and peak responses attained at 1 log higher peptide concentration (1 IM vs. 0.1 IM) (Fig. 4). This difference, observed in two independent experiments comparing 12 gag-Tg and 11 WT mice, was reproducible and highly statistically significant ($p \leq 0.001$).

Cytotoxic activity *in vitro* of gag₈₅₋₉₃-specific CD8⁺ T cells

The ability of the lower-avidity CD8⁺ T cell response induced by the p.DOM-gag DNA vaccine in gag-Tg mice to exhibit lytic activity against targets expressing the gag₈₅₋₉₃ epitope was tested. At day 36 after vaccination, splenocytes from WT or gag-Tg mice were stimulated *in vitro* with peptide for 6 days and lytic activity assessed in a ⁵¹Cr-release assay. CTL from either WT or gag-Tg mice, primed with p.DOM-gag, lysed EL4 target cells pulsed with gag₈₅₋₉₃ peptide but not a control peptide (Fig. 5A). CTL from either host also lysed FBL-3 leukemia

cells expressing endogenous FMuLV_{gag} antigen (Fig. 5A). No specific CTL activity was generated by culture of splenocytes *in vitro* with gag₈₅₋₉₃ peptide following vaccination with the control vaccine, p.DOM, and no lytic activity was observed against the NK-susceptible cell line YAC-1 (Fig. 5A).

Cytotoxic activity *in vivo* of gag₈₅₋₉₃-specific CD8⁺ T cells

Clearly, gag₈₅₋₉₃-specific lytic activity could be elicited following *in vitro* stimulation of CD8⁺ T cells that had been induced in gag-Tg mice. However, it was unknown whether the cells were capable of expressing such lytic activity within the *in vivo* tolerizing environment. To address this, WT and gag-Tg mice were vaccinated with either p.DOM-gag, the control DNA vaccine (p.DOM), or irradiated FBL-3 cells. At day 36, mice were injected intravenously with sex-matched, syngeneic splenocyte targets pulsed with either gag₈₅₋₉₃ peptide or control peptide that had been differentially labeled with CFSE to permit distinction between the two targets by flow cytometry. Twenty hours later, mice were sacrificed and the spleens analyzed for persistence of CFSE-labeled targets. WT mice that had previously been vaccinated with either p.DOM-gag or irradiated FBL-3 cells rapidly and efficiently lysed target splenocytes pulsed with the gag₈₅₋₉₃ peptide, as reflected by the clearance of >90% of the CFSE^{hi} targets by 20 h, but not those from the co-transferred population pulsed with the control peptide (CFSE^{low}) (Fig. 5B). Both populations of CFSE-labeled target cells survived in WT mice given the control DNA vaccine, p.DOM (Fig. 5B).

Gag-Tg mice vaccinated with p.DOM-gag also specifically eliminated the gag₈₅₋₉₃ peptide-pulsed target cells (Fig. 5B). The specific lytic activity in gag-Tg mice immunized with this DNA vaccine was slightly less than that observed in WT mice, but the difference did not reach statistical significance ($p=0.073$; Fig. 5C). Thus, for target cells pulsed with high concentrations of the peptide epitope, the reduced avidity of the CTL elicited in the transgenic mice did not markedly affect their performance. In contrast to WT mice, gag-Tg mice vaccinated with irradiated FBL-3 cells failed to lyse target cells labeled with the gag₈₅₋₉₃ peptide (Fig. 5B and C), confirming the inability of this approach to induce gag₈₅₋₉₃-specific CTL in these animals. Both populations of CFSE labeled target cells survived in gag-Tg mice that had received the control DNA vaccine (Fig. 5B and C).

Induction of gag₈₅₋₉₃-specific CTL by p.DOM-gag protects against FBL-3 leukemia growth *in vivo*

Although the lower-avidity gag₈₅₋₉₃-specific CTL induced in gag-Tg mice demonstrated the ability to lyse peptide-pulsed targets *in vivo*, their capacity to recognize and protect mice from leukemia *in vivo* was unknown. To address this, WT or gag-Tg mice were vaccinated with p.DOM-gag or the control vaccine (p.DOM) and then challenged at day 36 by intraperitoneal injection of 5×10^4 FBL-3 leukemia cells. Immunization with p.DOM-gag afforded significant protection from leukemia in WT mice, with ~95% surviving, compared to naive animals or those given the control vaccine (Fig. 6A). A low level of resistance ($\leq 25\%$) was occasionally observed in control WT groups, suggesting that injection of this dose of FBL-3 leukemia cells alone can lead to the spontaneous induction of natural protective immunity in WT mice (Fig. 6A).

Vaccination with p.DOM-gag also led to significant protection in gag-Tg mice (Fig. 6B). In these mice, there was no evidence for spontaneous induction of immunity following injection of FBL-3 cells, since all naive or control-vaccinated mice succumbed by day 19 (Fig. 6B). Thus, the lower-avidity gag₈₅₋₉₃-specific CTL response induced in gag-Tg mice by the p.DOM-gag vaccine is capable of mediating demonstrable protection from challenge with live FBL-3 leukemia cells *in vivo*. However, this response appears suboptimal compared to the responses elicited in WT mice, with only about one third of the gag-Tg mice vaccinated with

p.DOM-gag exhibiting prolongation of survival (Fig. 6B). It is unclear whether this detectable but reduced protection reflects primarily a qualitative or quantitative defect. One possibility is that the response might be further amplified by adding novel adjuvants.

Assessment of autoimmunity in vaccinated gag-Tg mice

Despite the significant degree of protection against leukemia afforded by vaccination of gag-Tg mice with p.DOM-gag, the presence of a population of activated gag₈₅₋₉₃-specific CD8⁺ T cells following DNA immunization could potentially result in autoimmune injury to hepatocytes expressing the FMuLV_{gag} protein. To assess this, blood was drawn from gag-Tg mice at day 36 following vaccination, and serum levels of the liver enzymes aspartate aminotransferase and alanine aminotransferase measured as indicators of liver injury. Mice were also sacrificed at this time point for blinded histological analysis of liver tissue. All animals appeared healthy with no evidence of increased levels of aspartate aminotransferase or alanine aminotransferase in the serum (data not shown). Histologic analysis of liver sections in vaccinated gag-Tg animals indicated that all portal tracts were normal, with normal biliary structures (Fig. 7). There was no inflammation in portal tracts or parenchyma, no hepatocyte degeneration or loss, and the normal sinusoidal architecture was present (Fig. 7). No autoimmune hepatocyte injury was evident, despite the relatively high levels of gag transcript expression observed in gag-Tg liver compared to FBL-3 tumor cells (Fig. 1).

Discussion

Effective vaccination of patients with cancer using tumor-associated antigens has several major problems to overcome. These include restrictions placed on the immune repertoire by expression of the tumor antigen on normal tissues and the obstacles to activating the residual repertoire to attack cancer cells and avoid autoimmunity.

The issue of tolerogenic pressure acting on the T cell repertoire has been widely investigated in mouse models. The most common strategy has been to combine two transgenic strains expressing either a targeted antigen or a T cell receptor (TCR) specific for an MHC class I-associated peptide derived from the antigen. Although these studies are confined to assessing the effects of antigen on a selected single-clonal T cell population, useful insights regarding the obstacles to inducing T cell responses have been derived. Many models involve T cells with high-affinity TCR, for which deletion is the usual outcome [34]. However, it is becoming clear that low-avidity T cells can survive both central and peripheral tolerance mechanisms provided that antigen expression is modest [7]. Development of autoimmune attack on tissues expressing the target antigen appears to follow the same rules of dependence on level of expression and T cell avidity [7]. Ideally, as is evident from clinical transplantation, the focus should be on antigens that are highly expressed by tumor cells but weakly expressed by normal cells.

A key challenge for the clinic is how best to activate the surviving lower-avidity T cells. In a transgenic model in which influenza hemagglutinin (HA) is expressed in pancreatic islet β -cells, low-avidity HA-specific CTL could be activated by immunization/infection with HA-expressing virus [35]. Such CTL were capable of recognizing a tumor expressing high levels of the HA antigen without injuring the pancreas. Further study of the fate of low-avidity transgenic CD8⁺ T cells in this and a related model has revealed that the level of antigen is a critical determinant, and that passively transferred CD8⁺ T cells ignored low levels of antigen in normal pancreatic tissue, but could recognize higher levels of antigen expressed in malignant pancreatic islet β -cells. However, these T cells exhibited limited anti-tumor activity unless antigen-specific CD4⁺ Th cells were provided and both T cell subsets were activated together by vaccination/infection with influenza virus [36].

Unfortunately, the repeated use of viral vectors is not possible due to induction of blocking immunity against viral proteins [37], a key disadvantage given that repeated vaccination is likely to be required for cancer patients. Even if blocking antibody can be avoided, viral vectors can lead to domination of the CTL hierarchy by virally derived epitope-specific responses, to the detriment of immune induction against lower-affinity tumor-derived epitopes encoded within the vaccine [38,³⁹]. Considering these requirements, we have explored a DNA fusion vaccine format that affords concurrent heterologous T cell help for effective CTL induction *in vivo*, avoiding the use of viral vectors or adoptive transfer. The design focuses immunity onto the encoded epitope and, when combined with electroporation at boosting, can achieve high levels of CTL [14].

Using this strategy, our aim was to induce CTL against a model tumor antigen that was also a self protein continually expressed in a large peripheral organ, but in which the T cell repertoire was unmanipulated and therefore polyclonal, as this represents a typical setting for many human cancers. We chose the gag-Tg mice since the antigen is mainly expressed in the liver, but lower levels are detectable in the thymus, and there is clear evidence for tolerance [32, ³³]. Furthermore, in a double-transgenic mouse expressing both gag in the liver and a TCR in CD8⁺ T cells specific for the gag₈₅₋₉₃ epitope [33], T cell numbers were reduced and those remaining failed to proliferate or produce IL-2 in response to antigen [40]. Although our DNA vaccine was operating within a polyclonal T cell repertoire, it is likely that it had to contend with a similar combination of deletional and regulatory influences in the gag-Tg mice, and the challenge was to activate the residual repertoire.

The DNA vaccine was able to mobilize an epitope-specific CD8⁺ T cell response capable of proliferation, cytokine production, and killing of target FBL-3 leukemia cells *in vitro* and *in vivo*. The provision of heterologous T cell help within the vaccine was aimed to circumvent tolerance to tumor antigens, which may be more profound in the CD4⁺ T cell arm. Interestingly the cellular vaccine failed to induce a detectable CD8⁺ T cell response against this dominant epitope in the transgenic mice. Since the potential response is clear, the weakening of the cellular vaccine is likely due to an inability to generate a tumor-derived CD4⁺ T cell response, possibly due to the activity of Treg. It is known that effective CD4⁺ T cell help is critical for inducing and maintaining an effective CTL response [24-²⁶]. One question is whether true tumor-specific T cell help might be required for long-term maintenance of CD8⁺ T cells [41]. In fact, our experience has been that challenge with tumor cells can expand tumor-specific CD8⁺ T cells that have previously been primed by DNA fusion gene vaccination [27]. Nonetheless, we are investigating the effect on CD8⁺ T cell priming of also encoding tumor-derived CD4⁺ T cell epitopes within our DNA vaccine and exploring the role of Treg. However, the main point of our current study is that, in contrast to the cellular vaccine, the DNA fusion vaccine is able to activate the residual CD8⁺ T cell repertoire and that these T cells can carry out their effector functions *in vivo*.

Epitope-specific CTL appear able to kill target tumor cells effectively, although targeting multiple epitopes might be even more potent [42], and avoid escape via epitope loss [43]. Vaccines encoding whole antigen cDNA appear to offer this potential, with the added advantage of being suitable for all patients regardless of MHC class I haplotype. However, placing an antigenic epitope at the C terminus of the FrC domain (DOM) promotes more efficient induction of CD8⁺ T cells compared with using native cDNA [27], and immunodominance argues against delivering multiple epitopes within the same vaccine [44-⁴⁶]. Instead, multiple vaccines could be used, each targeting a distinct tumor epitope, with delivery of the second vaccine into a separate site to avoid competition [45,⁴⁶]. This integrated approach could compensate for the loss of T cell repertoire against individual epitopes.

Following vaccination, the residual gag-specific CD8⁺ T cell repertoire surviving in the tolerized mice clearly retain the capacity to kill tumor cells *in vivo*. Unfortunately, the highly aggressive nature of the FBL-3 tumor makes it unsuitable to model therapeutic efficacy since even a dose of 100 FBL-3 tumor cells per gag-Tg animal leads to death at day 12–14 (data not shown). However, we have established previously that the vaccine design can be used therapeutically, using a less aggressive tumor model that allows the immune system time to respond to vaccination [14].

Once effector CTL are induced, the balance between tumor destruction and autoimmunity depends at least in part on the relative levels of peptide expressed [7], and on the accessibility of each target cell population to effector CTL infiltration [47]. Leukemic cells tend to be in blood or lymphoid tissues and access to effector CTL seems assured. Our data indicate that in this tolerance model, where expression of antigen in the thymus appears to cause deletion of the repertoire [32,³³], our DNA vaccine can activate residual gag-specific CTL able to lyse leukemia cells exhibiting a relatively low level of gag transcript expression. The vaccine design elicits no tumor-specific CD4⁺ T cells, thereby potentially protecting antigen-expressing organs from entry of CTL, which is likely to depend on cytokine-producing CD4⁺ T cells at the site [48]. Also, since these CD4⁺ T cells are targets for regulation, the vaccine may circumvent suppression first by utilizing the microbial repertoire and then by evading tumor-specific regulatory mechanisms. It is possible that the cellular vaccine containing irradiated tumor cells could elicit CD4⁺ T cell responses, but this was not investigated due to the lack of well-characterized gag-derived Th cell epitopes. For antigens expressed in the liver, there may also be other factors operating, such as an intrinsic resistance of the liver to autoimmune damage [32,^{47,49}]. Previous attempts to promote autoreactivity in this model by combining viral infection of the liver with transfer of gag-specific CTL were unsuccessful.

Control of tumor may require continuing surveillance by anti-tumor CTL. Once induced, their ability to survive will be influenced by the level, mode and persistence of expression of tumor antigen, together with the presence or absence of inflammatory mediators. Each model can provide only a partial view, and the best test of performance of the DNA fusion vaccines will therefore be in patients with cancer, with clinical trials now underway [21].

Materials and Methods

Cells

FBL-3 is a Friend virus-induced erythroleukemia of B6 origin that expresses FMuLV *gag*- and *env*-encoded products and MHC class I molecules [50]. EL4 is a T cell lymphoma derived from C57BL/6N mice, and YAC-1 is an NK-susceptible T cell lymphoma originating from the A/Sn strain. All cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS (Life Technologies, Paisley, UK), 1 mM sodium pyruvate, 2 mM L-glutamine, non-essential amino acids (1% of 100x stock), 25 mM HEPES buffer and 50 μM 2-mercaptoethanol (complete medium).

Peptides

The H-2D^b-restricted gag peptide gag_{85–93} derived from FMuLV_{gag} (CCLCLTVFL) and the FrC-derived Th peptide p30 (TT_{947–967}; FNNFTVSFWLRVPKVSASHLE) have been described previously [31,⁵¹]. Peptide controls included the H-2D^b-restricted HY peptide (WMHHNMDLI) derived from the *Uty* gene [52] and a TT-derived H-2K^b-restricted peptide (SNWYFNHL) which is not encoded within these DNA vaccines [22]. Peptides were synthesized commercially and supplied at >95% purity (PPRL, Southampton, UK). Gag_{85–93} peptide stocks (5 mM) were dissolved in DMSO, and all other peptide stocks (1 mM) were dissolved in water. Stocks were stored at –20°C.

DNA vaccines

DNA vaccine design is indicated in Fig. 2. Construction of a DNA vaccine (p.DOM) containing the gene encoding the first domain (DOM; TT_{865–1120}, 256 amino acids plus leader sequence of 19 amino acids) of FrC (TT_{865–1316}) from TT has been described [22]. The p.DOM vaccine was used as a template to construct p.DOM-gag, which encodes the immunodominant H-2D^b-restricted FMuLV_{gag} CTL motif (gag_{85–93}) fused to the C terminus of DOM. This was achieved by PCR amplification of DOM using the forward primer 5'-TTTAAAGCTTGCCGCCACCATGGGTTGGAGC-3' and the reverse primer 5'-TTTTGCGGCCGCTTACAGAAAAACAGTCAAACAGAGACAACAGTTACCCCAGAGTCACGCAGGAA-3', which fuses the gag_{85–93}-encoding sequence to the 3' terminus of DOM. The resulting PCR fragment was cloned into the expression vector pcDNA3 (Invitrogen, San Diego, CA). Both DNA constructs encode a leader sequence derived from the V_H of the IgM idiotype of the BCL1 tumor. DNA vaccine stocks were prepared and verified as previously described [29].

Mice and vaccination protocol

The B6 gag-Tg model, in which the gag protein from FMuLV is expressed under the control of the mouse albumin promoter in the liver, has been described previously [32,33,40]. For DNA immunization, WT B6 or gag-Tg mice, bred in house, were vaccinated at 6–12 wk of age with a total of 50 µg DNA in saline injected into two sites in the quadriceps muscles on day 0; mice were anesthetized and administered booster injections together with electroporation at day 28, as described previously [14]. For cellular immunization, mice were injected with 1×10⁷ irradiated (10 000 rad) FBL-3 leukemia cells intraperitoneally on days 0 and 28. Animal experimentation was conducted within UK Coordinating Committee for Cancer Research (London, UK) guidelines, under Governmental license.

ELISPOT assay

Following priming (day 0) and booster injections (day 28), splenocytes were harvested on day 36 and vaccine-specific responses were assessed *ex vivo* by ELISPOT assay for IFN-γ secretion (BD ELISPOT Set; BD PharMingen, San Diego, CA), as described previously [28]. Splenocytes were incubated with either the H-2D^b-restricted gag_{85–93} peptide for 24 h to assess CD8⁺ T cell responses or the p30 peptide (derived from the FrC fusion domain, DOM) to assess vaccine-induced CD4⁺ T cell responses. Triplicate wells were tested with a range of gag_{85–93} peptide concentrations; control samples were incubated without peptide. The reducing agent tris(2-carboxyethyl) phosphine hydrochloride (TCEP; Pierce Biotechnology, Rockford, IL), which has been shown to enhance the antigenicity of cysteine-containing synthetic peptides [53], was included in each microtiter well (200 µM) during the incubation stage, as the gag_{85–93} epitope with three cysteines is otherwise very insoluble *in vitro*.

To compare the frequency of T cells responding to different concentrations of the gag_{85–93} peptide, as a measure of T cell avidity, baseline ELISPOT responses without peptide were subtracted and the number of spot-forming cells (SFC)/million splenocytes calculated as a percentage of the maximum SFC/million splenocytes for each individual mouse. The data were pooled within each experimental group to calculate the mean ELISPOT response as a percentage of the maximum response observed for each peptide concentration.

Generation and assay of gag_{85–93}-specific CTL

To assess gag_{85–93}-specific CTL responses, vaccinated mice were sacrificed at day 36 and single-cell suspensions made from individual spleens in complete medium. Splenocytes were washed and resuspended in 15 mL complete medium at 3×10⁶ cells/mL, together with recombinant human IL-2 (20 IU/mL; Perkin-Elmer, Foster City, CA), gag_{85–93} peptide (0.01

μM) and 200 μM TCEP [53]. Following 6 days stimulation *in vitro* (37°C, 5% CO₂), cytolytic activity of the T cell cultures was assessed by standard 5-h ⁵¹Cr-release assay as previously described [22], with target cells that were labeled with peptide/⁵¹Cr in the presence of 200 μM TCEP for 1 h at 37°C. Targets included EL4 cells pulsed with gag₈₅₋₉₃ peptide or control peptide (Uty), FBL-3 leukemia cells or NK-sensitive YAC-1 cells.

***In vivo* cytotoxicity assay**

Splenocytes were harvested from WT and gag-Tg mice ($2 \times 10^7/\text{mL}$ in PBS) and cells from each strain were pulsed with 5 μM gag₈₅₋₉₃ peptide or control peptide (SNWYFNHL) for 30 min at 37°C in the presence of 200 μM TCEP and washed in PBS. The gag- and control peptide-pulsed cells were then incubated with 5 μM or 0.5 μM CFSE (Molecular Probes, Invitrogen), respectively, at room temperature for 8 min in the dark, and FBS (final concentration 20%) was added to quench the labeling reaction. After washing, syngeneic cells were mixed together, resuspended in PBS, and 2×10^7 cells in 0.1 mL injected intravenously to each sex-matched syngeneic recipient. Splenocytes were harvested from individual recipients after 20 h and, following lysis of RBC, cells expressing CFSE analyzed by FACSCalibur (BD Biosciences, San Diego, CA).

Tumor challenge

Mice were challenged 8 days after the booster immunization (day 36 following priming) by intraperitoneal injection of 5×10^4 FBL-3 leukemia cells in PBS. Mice were monitored daily and euthanized on detection of tumor development, in accordance with UK Coordinating Committee for Cancer Research guidelines.

***Ex vivo* intracellular IFN- γ assay**

To assess priming of gag₈₅₋₉₃-specific CD8⁺ T cells, mice were culled at day 36 following immunization, and splenocytes processed for detection of intracellular IFN- γ . Viable splenocytes were selected by density centrifugation and B cells removed using mouse pan B DynabeadsJ (Invitrogen). Cells were incubated for 4 h at 37°C in 96-well plates (1×10^6 cells/well) in complete medium together with 200 μM TCEP, 10 IU/well human recombinant IL-2, 1 μM gag₈₅₋₉₃ peptide or control peptide (SNWYFNHL) and 1 μL /well Golgi Plug (BD Biosciences). Following incubation, samples were processed to label surface CD8 and intracellular IFN- γ , as previously described [27], and analyzed by FACSCalibur.

Analysis of autoimmune injury

Following priming (day 0) and booster vaccinations (day 28), groups of B6 and gag-Tg mice were euthanized on day 36 to assess autoimmune injury. Control, naive groups received no vaccinations. Liver samples were fixed in formaldehyde, paraffinembedded, sectioned and stained with hematoxylin/eosin. Coded specimens were analyzed by a liver pathologist in a blinded manner for inflammation and lymphocyte infiltration using a Zeiss Axioskop 2 MOT microscope (Carl Zeiss, Oberkochen, Germany) and Zeiss Plan-NEOFLUAR 10x/0.30 objective lens. Images were recorded using a Zeiss AxioCam camera and Zeiss Axiovision 4 software with white balance correction provided by GNU Image Manipulation Program (<http://www.gimp.org>) and processed with CorelDraw Graphics Suite 12 (Corel, Ottawa, Canada).

Quantitative analysis of gag RNA expression

The FMuLV_{gag} open reading frame encodes two alternative translational products: Pr⁶⁵_{gag} and gPr⁷⁵_{gag}. The higher-molecular-weight protein gPr⁷⁵_{gag} has an additional 98-amino-acid N-terminal signal sequence [54,55] within which the target immunodominant gag₈₅₋₉₃ epitope is located [31]. The major protein species generated in FBL-3 tumor cells is Pr⁶⁵_{gag} [32,54].

The gag transgene present in gag-Tg mice was designed to optimize expression of only gPr⁷⁵gag [32,⁵⁴].

To assess gPr⁷⁵gag RNA expression, total RNA was extracted from FBL-3 cells, EL4 cells, liver or thymus biopsies from gag-Tg and WT mice using RNeasy Plus mini kit (QIAGEN, Valencia, CA). Total RNA was reverse-transcribed using an oligo(dT) primer and a First-Strand cDNA Synthesis kit (Amersham Pharmacia Biotech). The cDNA was used for all subsequent qRT-PCR assays, using Taqman Universal PCR master mix (Applied Biosystems, Foster City, CA) together with a primer/probe combination to detect either gPr⁷⁵gag gene expression (forward primer: 5'- GGCCGACTAGCTCTGTACCT- 3'; reverse primer: 50- GGTGCACCAAAGAGTCCAA- 3'; probe 5'-FAM-CGGGTGTCTCGAACCCGTCATAMRA-3') or endogenous mouse β -actin gene expression (Applied Biosystems). The gag primers were designed to only amplify product from gPr⁷⁵gag, containing the gag₈₅₋₉₃ epitope. All reactions were performed in triplicate using the ABI PRISM 7500 Sequence detection System (Applied Biosystems) according to the following thermal cycle protocol: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 90 s. The β -actin transcript level was used to normalize gene expression levels.

Statistical analysis

Experimental groups were compared using an unpaired, two-tailed *t*-test. Survival curves were compared using the χ^2 log-rank test. Experimental groups were considered significantly different from control groups if *p*<0.05.

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Abbreviations

B6	C57BL/6
FMuLV	Friend murine leukemia virus
FMuLV _{gag}	Friend murine leukemia virus gag protein
FrC	fragment C
gag-Tg	FMuLV _{gag} -transgenic
qRT-PCR	quantitative RT-PCR
SFC	spot-forming cells
TCEP	tris(2-carboxyethyl) phosphine hydrochloride
TT	tetanus toxin

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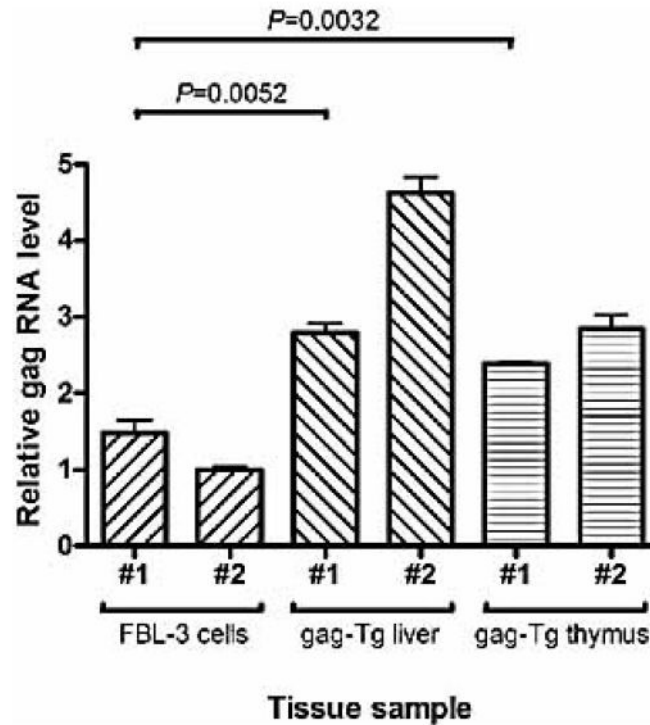


Figure 1.

Relative levels of gag RNA expression in FBL-3 tumor cells compared to liver and thymus cells from gag-Tg mice. Total RNA was extracted from FBL-3 cells, thymus or liver biopsies from gag-Tg mice; samples were isolated from either two independent FBL-3 cell cultures (#1, #2) or two individual mice (#1, #2). Following cDNA synthesis, qRT-PCR was used to compare the relative levels of gag gene expression between samples; qRT-PCR reactions were run in triplicate for each sample and combined; error bars: SEM. -actin transcript amplification was detected in all cDNA samples and was used to normalize gene expression levels.

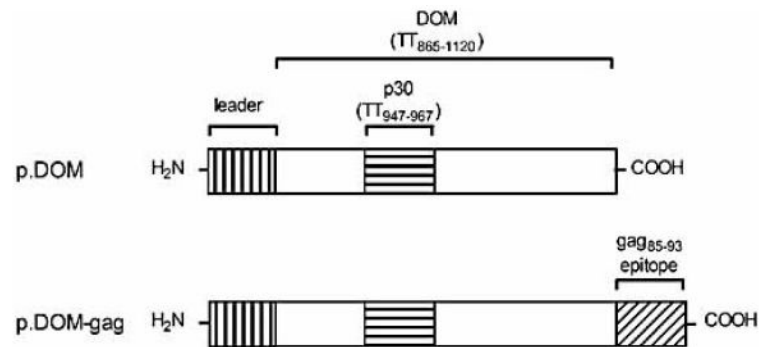
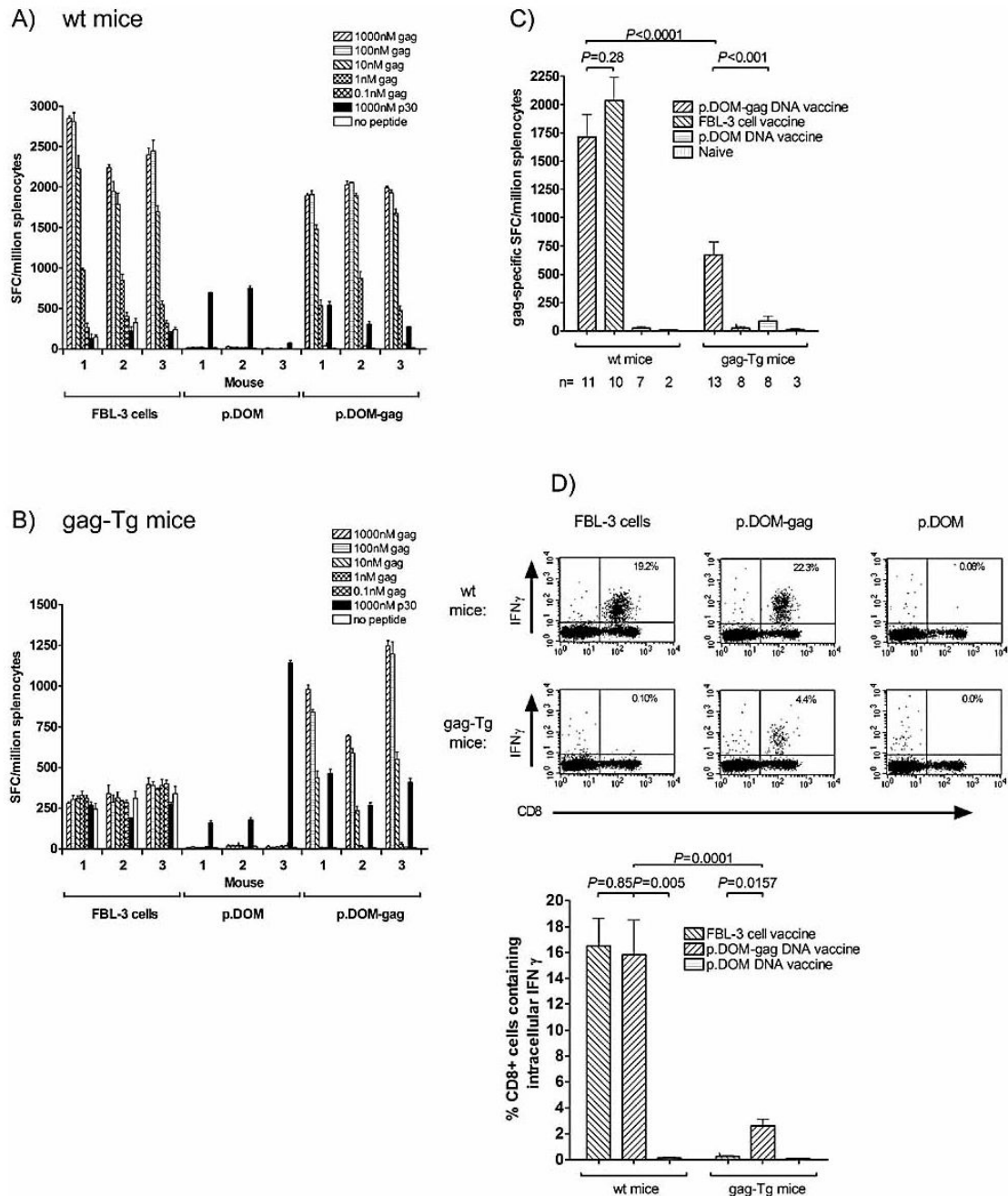


Figure 2.

Schematic diagram indicating DNA fusion vaccine design. The control vaccine p.DOM contains the sequence encoding the N-terminal domain (DOM) of FrC from TT, including the p30 CD4⁺ Th epitope. Sequence coordinates relative to the original TT sequence are indicated. p.DOM-gag contains DNA sequence encoding the immunodominant H-2D^b-restricted gag₈₅₋₉₃ CTL epitope derived from FMuLV_{gag} ligated in-frame to the C terminus of DOM. Each vaccine includes a leader sequence at the N terminus. Vaccine sequences were assembled and inserted into the commercial vector pcDNA3 using *Hind* III and *Not* I restriction sites.

**Figure 3.**

Ex vivo detection of gag-specific T cells in WT and gag-Tg mice following DNA vaccination. WT and gag-Tg mice were immunized with p.DOM-gag or p.DOM DNA vaccines, or irradiated FBL-3 cells, as indicated, on days 0 and 28; DNA booster injections at day 28 were administered, together with electroporation. Splenocytes were harvested from individual mice at day 36. (A, B) The numbers of SFC secreting IFN- were assessed *ex vivo* by ELISPOT assay. Splenocytes were incubated with gag₈₅₋₉₃ peptide to assess the epitope-specific CD8 $^{+}$ T cell response, and with the p30 peptide to assess CD4 $^{+}$ T cell responses to the DOM component of the vaccine. Test samples were set up in triplicate; baseline responses without peptides are indicated. The data indicate mean SFC/million splenocytes and the SEM (error bars).

Representative data from one of two identical experiments are shown. (C) The mean number of gag₈₅₋₉₃ epitope-specific SFC was compared between WT and gag-Tg mice. Responses to 1 M gag₈₅₋₉₃ peptide were pooled from individual mice; data are expressed as mean SFC/million splenocytes, together with the SEM. The number (*n*) of animals pooled per group is indicated. (D) The frequency of gag₈₅₋₉₃-specific CD8⁺ T cells producing IFN- was assessed *ex vivo* by intracellular cytokine staining. Representative flow cytometric plots from individual mice are shown (upper panel). Data were pooled from individual mice (lower panel) to indicate the mean percentage of CD8⁺ T cells producing IFN- in response to gag₈₅₋₉₃ peptide; error bars: SEM.

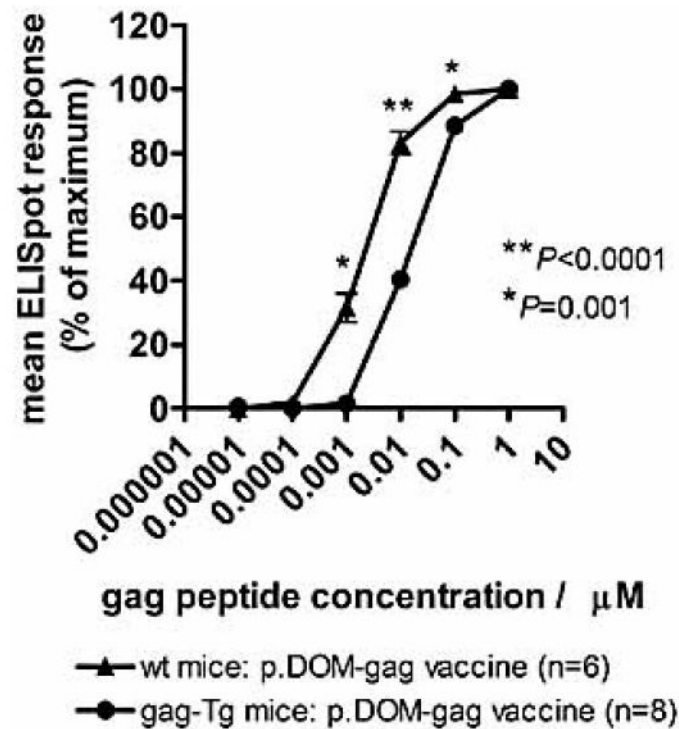


Figure 4.

DNA vaccination induces lower-avidity gag₈₅₋₉₃-specific CTL in gag-Tg mice compared to WT mice. WT and gag-Tg mice were immunized with p.DOM-gag on days 0 and 28; booster injections at day 28 were administered, together with electroporation. Splenocytes were harvested from individual mice at day 36 and the numbers of SFC secreting IFN- were assessed *ex vivo* by ELISPOT assay. Splenocytes were incubated with varying concentrations of gag₈₅₋₉₃ peptide to assess the epitope-specific CD8⁺ T cell response; test samples were set up in triplicate for each peptide concentration. Baseline responses without peptide were subtracted and the number of SFC/million splenocytes was calculated as a percentage of the maximum number of SFC/million splenocytes responding per mouse; data were then pooled for each experimental group to calculate the mean ELISPOT response, together with the SEM (error bars). Representative data from one of two identical experiments are shown.

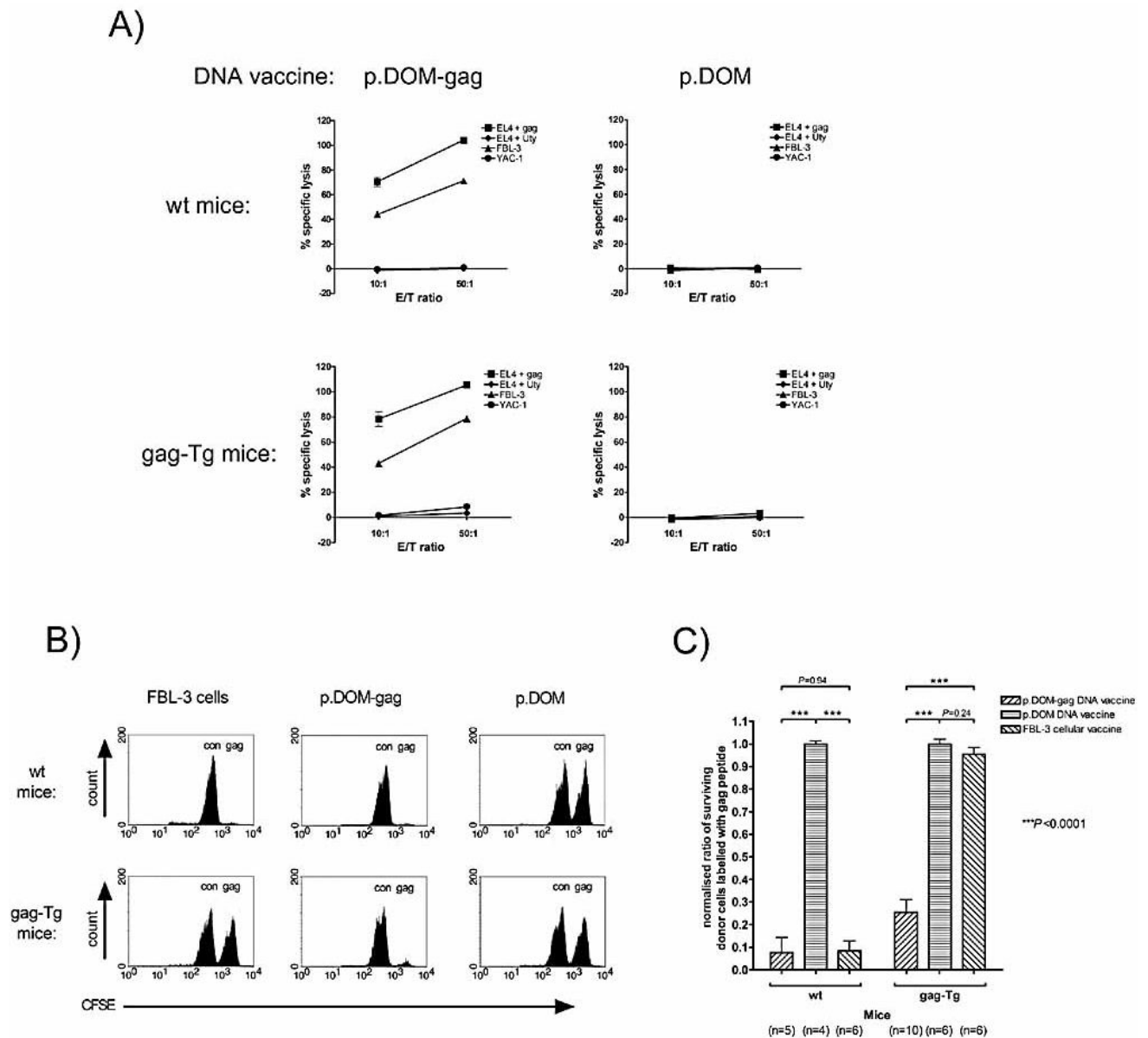


Figure 5.

DNA vaccination of gag-Tg mice induces gag₈₅₋₉₃-specific effector CTL capable of *in vitro* and *in vivo* cytolytic activity. WT and gag-Tg mice were immunized with p.DOM-gag or p.DOM DNA vaccines, or irradiated FBL-3 cells, as indicated, on days 0 and 28. (A) At day 36, splenocytes were cultured with 0.01 M gag₈₅₋₉₃ peptide for 6 days *in vitro*, prior to measuring CTL activity by standard ⁵¹Cr-release assay. Targets were EL4 cells pulsed with gag₈₅₋₉₃ peptide or control peptide (Uty), FBL-3 leukemia cells expressing endogenous FMuLV_{gag} antigen, or the control cell line YAC-1. Representative data from individual mice are shown; data from one of two identical experiments are shown; error bars: SEM. (B, C) At day 36 following vaccination, mice were infused, by intravenous injection, with a 1:1 mixture of target splenocytes: one population of cells had been pulsed with gag₈₅₋₉₃ (gag) peptide while the other had been pulsed with control (con) peptide (SNWYFNHL); each population was labeled with a different concentration of CFSE (gag₈₅₋₉₃ peptide: CFSE^{hi}; control peptide:

CFSE^{low}). Splenocytes were harvested 20 h later and the frequency of each CFSE-labeled target cell population determined by flow cytometric analysis. (B) Representative flow cytometric profiles from individual mice. (C) The ratio of gag₈₅₋₉₃- to control peptide-pulsed cells surviving in each recipient was calculated. The ratios were normalized, by assuming that the mean ratio was 1:1 in mice given the control vaccine (p.DOM), and pooled to calculate the mean proportion of surviving donor cells pulsed with the gag₈₅₋₉₃ peptide, together with the SEM (error bars). Data are pooled from two of two identical experiments, each giving similar results; the number (*n*) of animals pooled per group is indicated.

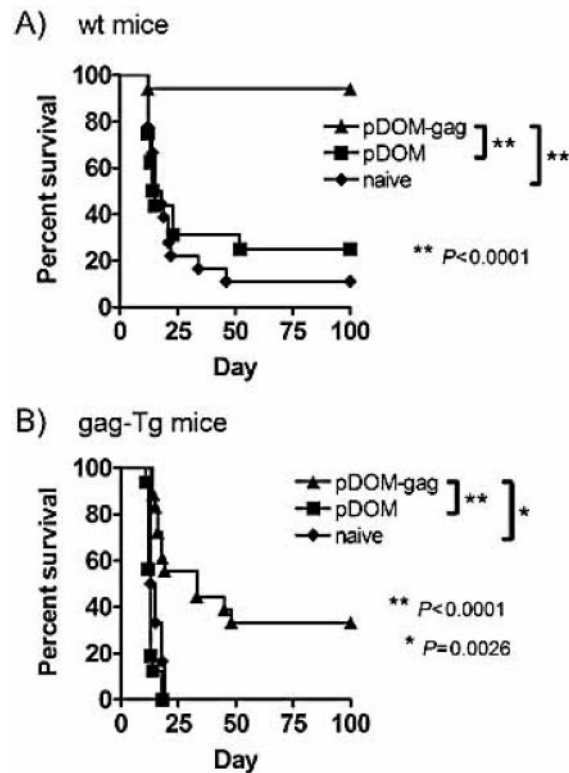


Figure 6.

DNA vaccination of gag-Tg mice induces gag₈₅₋₉₃-specific effector CTL able to protect mice from FBL-3 leukemia. (A) WT and (B) gag-Tg mice were immunized with p.DOM-gag or p.DOM DNA vaccines as indicated, on days 0 and 28; DNA booster injections at day 28 were administered, together with electroporation. Naive (unimmunized) mice served as controls. At day 36 mice were challenged by intraperitoneal injection of FBL-3 leukemia cells, and tumor development monitored. Survival data in each panel are pooled from two of two identical experiments, each giving similar results.

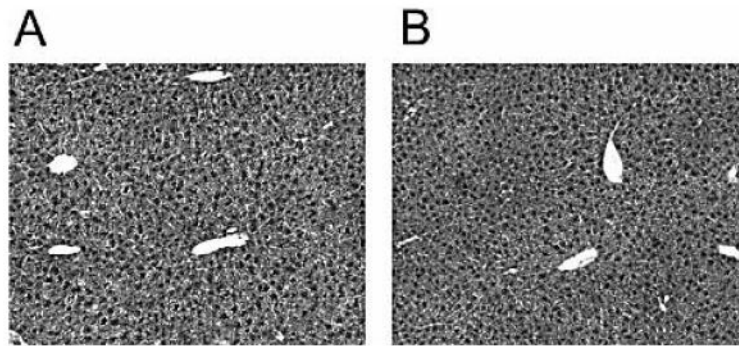


Figure 7.

Absence of autoimmune liver injury following DNA vaccination of gag-Tg mice. Gag-Tg mice were immunized with p.DOM-gag on days 0 and 28; DNA booster injections at day 28 were administered, together with electroporation. Unimmunized mice served as naive controls. Mice were euthanized at day 36 and liver samples were fixed in formaldehyde, paraffin-embedded, sectioned and stained with hematoxylin/eosin. Representative sections from gag-Tg mice immunized with p.DOM-gag (A) or naive gag-Tg controls (B) are shown; magnification, $\times 10$.

Table I

Ex vivo detection of vaccine-specific T lymphocytes in wt and gag-Tg mice by IFN γ ELISpot assay.

Vaccine	Peptide *	Vaccine-specific responses: [†]	
		wt mice	gag-Tg mice
p.DOM-gag	gag ₈₅₋₉₃	11/11 (100%)	12/13 (92%)
	p30	10/11 (91%)	13/13 (100%)
p.DOM	gag ₈₅₋₉₃	0/7 (0%)	0/8 (0%)
	p30	7/7 (100%)	6/8 (75%)
Irradiated FBL-3 cells	gag ₈₅₋₉₃	10/10 (100%)	0/8 (0%)
	p30	0/10 (0%)	0/8 (0%)
None	gag ₈₅₋₉₃	0/2 (0%)	0/3 (0%)
	p30	0/2 (0%)	0/3 (0%)

* Splenocytes incubated with 0.1 μ M gag₈₅₋₉₃ peptide or 1 μ M p30 peptide.

[†] Data presented as: number of positive responders/total mice tested (% responding).